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Immunoreactive and Bioactive Atrial Natriuretic Peptide in the Carp Heart

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ABSTRACT—Almost all of the secretory garnules in both the atrial and ventricular cardiocytes were immunolabeled. Chromatographic analysis of atrial and ventricular extracts indicated that the major component of immunoreactive ANP was a peptide with high molecular weight, which appeared to be equivalent to γ-ANP. These results suggest that ANP-like material is stored in the secretory granules in the atrial and ventricular cardiocytes of the carp, mainly as γ-type ANP. Furthermore, the atrial extracts showed a clear vasodepressor activity in quail, which disappeared after preincubation of the extract with excess antibodies raised against α-human ANP. Thus, immunoreactive and bioactive ANP in carp hearts may be identical.

INTRODUCTION

It is now well established that the atrial cardiocytes in mammals produce a family of atrial natriuretic peptides (ANPs), which seem to be involved in regulation of the body fluid balance and blood pressure [for review, see 1]. In several mammalian species, it has been demonstrated that ANP is stored in secretory granules in the atrial cardiocytes in the form of γ-ANP, which has 126 amino acid residues [2–4], and its C-terminal 28-residue peptide, α-ANP, circulates in blood [3, 5]. In the human atrium, α- and β-ANP (antiparallel dimer of α-ANP) have been identified in addition to γ-ANP [3]. In non-mammalian vertebrates, immunoreactive ANP (ir-ANP) was demonstrated in the atrial and ventricular cardiocytes of two species of reptiles, and of several species of anurans and fishes by immunohistochemical techniques or radioimmunoassay (RIA) using antisera against mammalian ANPs [6–15]. Further, the chemical structure of ANP has been determined in the chicken [16], two species of anurans [17, 18] and the Japanese eel [19]. Eel ANP has 59% sequence homology to mammalian (human or rat) ANP. Previously, we surveyed ANP immunoreactivity in the atria and ventricles by immunohistochemical techique and estimated the concentration of ir-ANP in heart tissues and plasma by RIA, using antiserum specific for α-human ANP in a variety of fishes [15]. In extending these findings, the present investigation was performed to localize ANP in the cardiocytes at the electron-microscopic level, and then, it was characterized by a combination of high-performance gel-permeation chromatography and RIA. Finally, the biological activity of the ir-ANP from carp atrial extracts was examined by the vasodepressor effect in the Japanese quail.

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MATERIALS AND METHODS

Animals

The carp, *Cyprinus carpio* (18–25 cm in total length), of both sexes were obtained from a commercial source and kept in groups of 10 in 400-liter aquaria (20–22°C) for at least 3 days before sac-

rifice. Fish were pithed, and the hearts were immediately dissected out and used for experiments.

Male Japanese quail, *Coturnix coturnix japonica*, 4 weeks of age, were purchased from a local dealer and kept individually under an 8L : 16D photocycle at 25±1°C for at least 2 weeks before use. Compound diet for quail (Nippon Haigo Shiryo, Yokohama) and water were available *ad libitum* until the day of experiment. The birds weighed 105±1 g (n=14) at the time of the experiment.

Immunocytochemistry

The atrium and ventricle of carp were cut into small pieces of less than 1 mm^3 and fixed at room temperature for 3 hr in a solution of 1.5% formaldehyde and 1.5% glutaraldehyde that contained 0.2% picric acid and was buffered with 0.1 M cacodylate buffer at pH 7.2. Thereafter, the tissues were stained "*en bloc*" with 1% uranyl acetate in 0.1 M sodium cacodylate, pH 7.2, at room temperature for 2 hr. After rinsing in the same buffer, the tissues were dehydrated through a graded ethanol series and propylene oxide and then embedded in Spurr's resin.

The post-embedding protein A-gold technique for thin sections was applied essentially as described by Bendayan and Zollinger [20]. Thin sections mounted on nickel grids were incubated in phosphate-buffered saline (PBS), pH 7.4, that contained 0.5% bovine serum albumin (0.5% BSA-PBS) for 30 min at room temperature. Each section was treated with a drop of α-human ANP-specific antiserum (Peptide Institute, Inc., Osaka) diluted 1 : 1000 in 0.5% BSA-PBS and incubated at 4°C overnight. After rapid washing in PBS, sections were incubated in a drop of protein A-gold complex diluted 1 : 20 in 0.5% BSA-PBS, for 30 min at room temperature. The grids were washed thoroughly in PBS, rinsed in distilled water and dried. The sections were then stained with lead citrate and examined with a JEOL JEM-100B electron microscope. Control reactions to demonstrate the specificity of immunogold labeling were carried out by replacing the solution of primary antibody with normal rabbit serum (1 : 1000). No gold particles were observed in the control sections, indicating that the immunogold labeling was specific under the parameters used. The anti-α-human ANP antiserum used in the immunocytochemical procedure was raised in rabbits and is known to have 55% cross-reactivity with α-rat ANP.

Chromatographic analysis

Extraction procedure The atrial and ventricular tissues were boiled for 10 min in 1 M acetic acid that contained 20 mM HCl to inactivate proteolytic activity. The boiled tissue was homogenized with a Polytron homogenizer and centrifuged at 15,000×g for 60 min at 4°C. The supernatant was stored at −30°C until chromatographic analysis.

High-performance gel-permeation chromatography (HP-GPC) The tissue extracts were applied to a TSK-GEL G2000 SW column (7.5×600 mm; Tosoh, Tokyo) and the column was eluted with 10 mM trifluoroacetic acid that contained 0.2 M sodium chloride and 30% acetonitrile as solvent at a flow rate of 0.3 ml/min. The eluates were lyophilized and dissolved in the assay buffer for RIA, which was 0.1 M Tris acetate, pH 7.4, containing 0.1% BSA and 1 mM Na_2EDTA. The recovery of synthetic α-human ANP added to heart extracts was about 80%.

RIA procedure Ir-ANP was determined by the

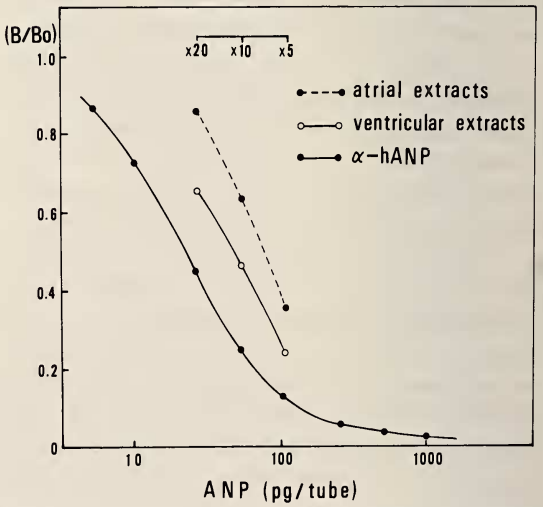

FIG. 1. A typical standard curve for α-human ANP and serial-dilution curves for carp atrial and ventricular extracts.

RIA method described previously [21, 22]. The antiserum was raised in New Zealand white rabbits against α-human ANP and was used at a final dilution of $1:4\times10^4$ to yield a maximum binding of approximately 35% of the total amount of [^{125}I]α-human ANP in the assay. Cross-reactivities with α-rat ANP and β-human ANP were 16% and 90% on a molar basis, respectively. The sensitivity of the RIA was 2 pg/tube with 50% displacement at 30 pg/tube. The dilution curves obtained with extracts from atrial and ventricular tissues were almost parallel to the standard curve for α-human ANP (Fig. 1).

Vasodepressor bioassay

Extraction procedure The atrial tissues were boiled for 10 min in 1 M acetic acid that contained 20 mM HCl to inactivate proteolytic activity. The mixture was homogenized, centrifuged at 3,000×g for 20 min at 5°C, and the supernatant was stored at −80°C. Subsequently, the thawed supernatant was passed through a conditioned, disposable extraction column (Sep-Pak C_{18}, Waters Associates, Milford, MA, USA), and trapped materials were eluted with 80% methanol in 0.1 M acetic acid. The eluate was dried under a stream of nitrogen, reconstituted in 0.9% NaCl and stored frozen at −25°C until use.

Bioassay procedure Mammalian ANPs are known to have the relaxant effect on the contractility of chick rectum [23]. In a preliminary experiment, however, extracts of carp heart had no effect on the precontracted chick rectum *in vitro*. Thus, a vasodepressor bioassay was performed using quail for carp ANP. For the bioassay, each quail was anesthetized by an intramuscular injection of urethane (1.5 g/kg), and a polyethylene tube was inserted into the right external jugular vein for injections and a second tube was inserted into the common carotid artery for measurements of arterial pressure. Procedures for cannulation and the experimental set-up have been described in detail elsewhere [24].

In order to examine whether or not ir-ANP and bioactive ANP in carp hearts were identical, ir-ANP was absorbed with antibodies to α-human ANP, and the effect of absorption was examined in the quail vasodepressor bioassay. A heart extract was mixed with aliquots of 0 to 100 μl of α-human ANP-specific antiserum (1:20, Peptide Institute, Inc.), and the volume was adjusted to 1.1 ml with 0.01 M phosphate buffer (pH 7.4). The mixture was incubated at 37°C for 1 hr, then at 4°C for 22 hr. It was then mixed with 3 mg of protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Tokyo) and incubated at 4°C for 50 min. The entire mixture was finally centrifuged at 4,200×g at 5°C for 20 min, and the resultant supernatant was injected into a quail after the dose-response relationship for α-human ANP had been examined. Injection volume was 0.2 ml for both extract and α-human ANP. Each injection was followed by flushing with 0.05 ml of saline. As controls, 33 μl of antiserum (1:20) and 1,067 μl of buffer were incubated together, the mixture was treated as described above, and the supernatant was injected into the quail. Further, in order to check the proteolytic activity in the antiserum, three mixtures were prepared. Each mixture contained 310 μl of extract and sufficient buffer to increase the total volume to 465 μl. The first mixture also contained 5 μl α-human ANP antiserum (1:20), and the second mixture also contained 5 μl normal rabbit serum (1:20). The third mixture contained extract and buffer only. These three mixtures were treated separately as described above, and the supernatant was injected into the quail. The anti-α-human ANP antiserum used in the bioassay procedure was the same as that used in the immunocytochemistry.

RESULTS

Immunocytochemistry The atrial cardiocytes contained many secretory granules near the nucleus and these granules were often also seen near the cell membrane. In the ventricle, most cardiocytes contained a few secretory granules, while some cells contained many granules near the nucleus. In both the atrium and ventricle, almost all of the secretory granules reacted with the α-human ANP-specific antiserum. No specific immunoreaction was observed in the cell matrix or on structures other than the secretory granules (Fig. 2a, b).

Chromatographic analysis Ir-ANP in the carp

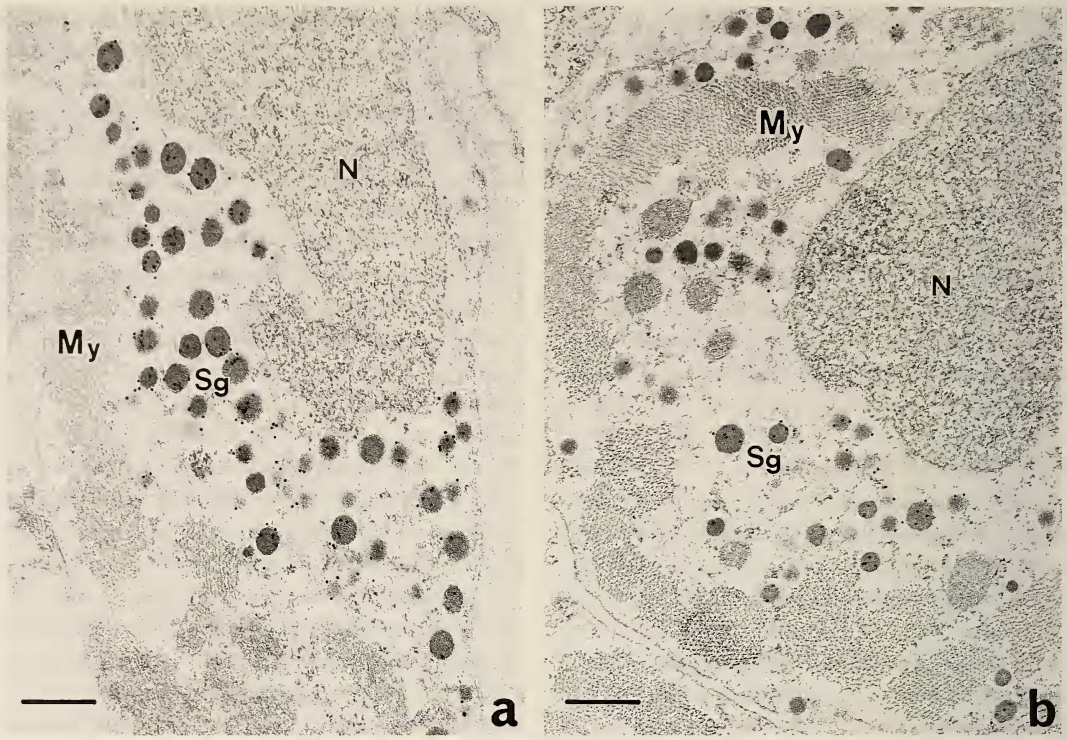

FIG. 2. Atrial (a) and ventricular (b) cardiocytes of *Cyprinus carpio* treated by the protein A-gold method, using antiserum against α-human ANP. Secretory granules (Sg) are labeled with gold particles. My, myofibrils; N, nucleus. $\times$20,000; bar, 0.5 μm.

atrial extracts consisted of three components (Fig. 3). The dominant peak was located near the position of elution of cytochrome *c*, with a molecular weight of 12.5 kDa. Two minor peaks were located at the positions of elution of synthetic β-human ANP and α-human ANP, respectively. The ir-ANP in the carp ventricular extracts generated a pattern similar to that generated by the atrial extract (Fig. 3).

Vasodepressor activity The carp atrial extracts decreased the arterial pressure and heart rate in quail as observed with human ANP (Fig. 4). After absorption of ir-ANP in the extracts with α-human ANP-specific antiserum, the vasodepressor activity was observed to decrease with increasing amounts of antiserum (Fig. 4). Incubation of the extracts with normal rabbit serum did not decrease the vasodepressor activity of the extracts.

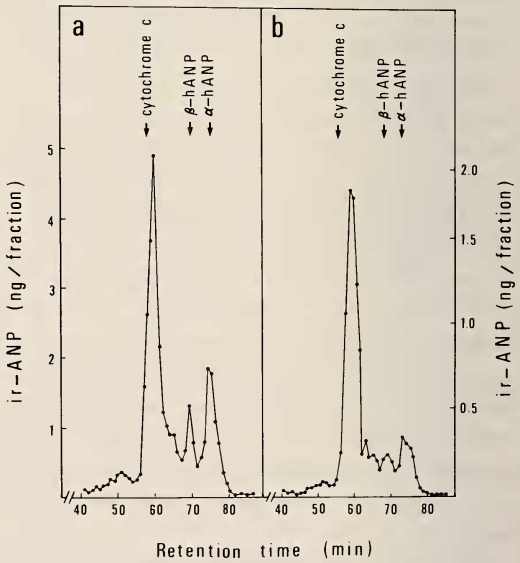

FIG. 3. Gel-permeation patterns for ir-ANP in extracts from carp atria (a) and ventricles (b). The arrows show the positions of elution of cytochrome *c* (12.5 kDa), β-human ANP and α-human ANP, respectively.

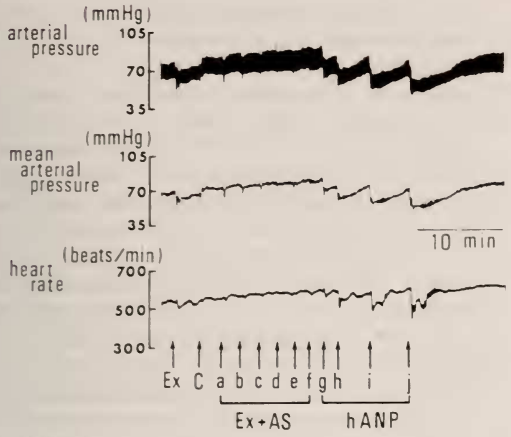

FIG. 4. Vasodepressor effects of the carp atrial extract (Ex) and α-human ANP (hANP) in a urethane-anesthetized quail (BW, 105 g). The depressor activity of the Ex decreased after incubation of Ex with increasing amounts of α-human ANP-specific antiserum (AS). C, AS alone (1:688). a-f, Ex incubated with AS. Final concentrations of the AS: 1:11,000 (a); 1:5,500 (b); 1:1,375 (c); 1:688 (d); 1:334 (e); and 1:220 (f). Doses of α-human ANP: 0.075 μg (g); 0.15 μg (h); 0.3 μg (i); 0.75 μg (j) per bird.

DISCUSSION

In the present study, almost all of the secretory granules in the ventricular cardiocytes, as well as those in the atrial cardiocytes, reacted with the α-human ANP-specific antiserum, as assessed by the protein A-gold technique. Thus, most granules in both the atrial and ventricular cardiocytes of the carp seem to contain ANP-like material. This result is in agreement with similar findings in two species of anurans [8, 10, 11], and a cyclostome [12], but the secretory granules were not always positive for human ANP in the Japanese eel [15, 25]. The discrepancy may be due to the different affinity of eel ANP to human ANP-specific antibodies.

In a previous study, we demonstrated that both the atrium and the ventricle of the carp contain the ir-ANP, which was estimated by RIA for human ANP as 44.6±3.5 ng/mg (n=5) and 2.6±0.4 ng/mg (n=4), respectively [15]. In extending these findings, the present study revealed that the major component of ir-ANP in atrial and ventricular extracts from the carp was a peptide with a molecular weight of about 12.5 kDa. This peptide may possibly be γ-ANP, since its molecular weight is roughly similar to that of mammalian γ-ANP [2]. In mammals, it has been well established that γ-ANP is a prohormone of the α-type peptide, the latter being the major circulating form of ANP. To date, peptides with similar molecular weight have been found to be the major components of ir-ANP in the atria of the chick and the turtle [9, 16] and in both the atria and the ventricles of *Rana ridibunda* [10, 11] and the Japanese eel [25].

In the carp atrial and ventricular extracts, two minor components were eluted at the positions of synthetic β-human ANP and α-human ANP, respectively. Although the possibility of proteolytic cleavage of the high-molecular-weight peptide during extraction cannot be completely excluded, the extraction procedure used in this study was designed such that any proteolytic activity in the homogenates would be inactivated. Thus, processing of the γ-type peptide may occur within the heart tissue. A similar result has been obtained in the Japanese eel [25].

In the present study, the carp atrial extracts showed vasodepressor activity in the quail, and the activity was diminished after incubation of extracts with α-human ANP-specific antiserum. This reduction in biological activity is not related to breakdown of the peptide by the proteolytic activity in the antiserum, since incubation of extracts with normal rabbit serum did not decrease the vasodepressor activity of the extracts. These results suggest that the vasodepressor activity is due to the ir-ANP in the extracts. Furthermore, it is possible that the ir-ANP lowers blood pressure in the carp as in the quail, since it was observed previously that ANP from the eel decreased arterial pressure in the eel as well as in the quail [19]. In agreement with our results, it has been reported that injection of homologous heart extracts or of mammalian ANP lowers blood pressure in the chicken [26], turtle [27] and toadfish [28]. In experiments *in vitro*, it was observed that heart extracts from the chicken, lizard, frog and trout exerted potent vasorelaxant effects on the rabbit aorta [13]. Taken together, all these results suggest that ANP is involved in the regulation of blood pressure in non-mammalian species, as is the

case in mammals [for review, see 1].

In summary, the present data demonstrate that both the atrial and ventricular cardiocytes of carp produce ANP-like material that is immunologically related to human ANP, and that both types of cardiocytes store this material in the secretory granules, mainly in the form of γ-type ANP. Furthermore, this material has vasodepressor activity in quail. Whether the carp ANP plays a physiological role in the regulation of blood pressure in carp remains to be determined.

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